Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al. Confirmation No.: 8515
Serial No.: 09/914,020 Art Unit: 1633
Filed: December 31, 2001 Examiner: J. Epps-Ford
For: *Methods and Compositions for Altering Mucus Secretion*

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT B

Vergeres et al. (1998) *"Binding of MARCKS (myristoylated alanine-rich C kinase substrate)-related protein (MRP) to vesicular phospholipids membranes,"* Biochem. J., 330, pp. 5-11.

Binding of MARCKS (myristoylated alanine-rich C kinase substrate)-related protein (MRP) to vesicular phospholipid membranes

Guy VERGÈRES[1] and Jeremy J. RAMSDEN

Department of Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland

The myristoylated alanine-rich C kinase substrate (MARCKS) protein family has two known members, MARCKS itself and MARCKS-related protein (MRP, also called MacMARCKS or F52). They are essential for brain development and are believed to regulate the structure of the actin cytoskeleton at the plasma membrane. Hence membrane binding is central to their function. MARCKS has been quite extensively characterized; MRP much less so. Despite the fact that MRP is only two thirds the size of MARCKS, it has hitherto been assumed that the two proteins have similar properties. Here we make a detailed study, including the effects of myristoylation, lipid composition, calmodulin and phosphorylation of the binding of MRP to phospholipid vesicles. We show that both the N-terminal myristoyl moiety and the central effector domain mediate binding. MRP behaves like MARCKS in the presence of neutral phospholipids. In contrast to MARCKS, however, the incorporation of 20% of negatively-charged phospholipids only marginally increases the affinity of myristoylated MRP. Co-operativity between the myristoyl moiety and the effector domain of MRP is weak and the protein has a significantly lower affinity for these vesicles compared with MARCKS. Furthermore, calmodulin or phosphorylation of the effector domain by the catalytic subunit of protein kinase C do not significantly decrease the binding of myristoylated MRP to negatively-charged phospholipid vesicles. Our results show that the mechanisms regulating the interactions of MARCKS and MRP with phospholipid vesicles are, at least quantitatively, different. In agreement with cellular studies, we therefore propose that MARCKS and MRP have different subcellular localization and, consequently, different functions.

INTRODUCTION

The members of the myristoylated alanine-rich C kinase substrate (MARCKS) family are widely distributed acidic, rod-shaped proteins essential for brain development and survival [1,2]. Changes in expression, protein kinase C (PKC)-dependent phosphorylation and subcellular localization of MARCKS proteins occur concomitantly with cellular events such as neurosecretion, mitogenesis, transformation, motility and phagocytosis [3,4]. The family comprises two members: MARCKS, a ubiquitous 32 kDa protein; and MARCKS-related protein (MRP, also called MacMARCKS or F52), a 20 kDa protein, predominantly expressed in brain and reproductive tissues [2,5,6].

MARCKS proteins contain a highly basic region, the effector domain (also the phosphorylation site domain or PSD). This domain is phosphorylated following activation of cellular PKC with phorbol esters [7], binds calmodulin (CaM) in the presence of calcium with high affinity (K_d < 20 nM) [8,9], and cross-links actin filaments *in vitro* [10]. It has been inferred that positively-charged residues mediate the binding of the effector domain to negatively-charged phospholipid membranes via electrostatic interactions. These interactions are presumably regulated by PKC, since phosphorylation of the serine residues decreases the affinity of the effector domain of MARCKS for membranes [11–13].

The N-terminal glycine residue of MARCKS proteins is myristoylated via a reaction catalysed by myristoyl CoA:protein N-myristoyl transferase (NMT) [3]. Studies with acylated peptides as well as with the intact protein show that the myristoyl group is involved in membrane binding [8,14–16]. Photolabelling of MARCKS and MRP with a photoprobe attached to the alkyl chain of phosphatidylcholine demonstrates that the myristoyl group is inserted in the bilayer [17]. Since the contributions of the myristoyl group and of the effector domain are not individually sufficient to firmly anchor MARCKS proteins to membranes, a model in which both domains act co-operatively has been proposed [18].

This model, the 'myristoyl-electrostatic switch', provides a rationale for the translocation of MARCKS observed following cellular activation of PKC by phorbol esters. In synaptosomes, the activation of PKC results in translocation of MARCKS from the membrane fraction to the cytosol [19]. Treatment of neutrophils with a chemotactic peptide is accompanied by transient translocation of MARCKS [20]. In macrophages that have spread on a substratum, MARCKS clusters at the cell-substratum interface of pseudopodia and filopodia and is co-localized with actin, vinculin and talin. Activation of PKC results in the displacement of MARCKS from the clusters [21].

Although the interactions of MARCKS proteins with membranes are evidently central to its function, the underlying molecular mechanisms are incompletely understood [18,22]. First, although there is strong evidence that PKC-dependent phosphorylation is a major regulator of the subcellular localization of MARCKS proteins, activation of PKC does not correlate with cytosolic localization of MARCKS proteins in myocytes [23], neuroblastoma cells [24], or macrophages [25]. Second, as unmyristoylated MRP can be 'hydrophobically-photolabelled', domains other than the myristoyl group must be inserted in the lipid membrane [17]. This conclusion was recently confirmed by a spectroscopic study with spin labels demon-

Abbreviations used: CaM, calmodulin; DTT, dithiothreitol; ECL, enhanced chemiluminescence; K_d^{eff}, effective dissociation constant; K_p molar partition coefficient; MARCKS, myristoylated alanine-rich C kinase substrate; MRP, MARCKS-related protein; myrMRP, myristoylated MRP; NMT, myristoyl CoA:protein N-myristoyl transferase; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; PKC, protein kinase C; PKM, catalytic subunit of PKC; unmyrMRP, unmyristoylated MRP.

[1] To whom correspondence should be addressed.

strating that hydrophobic residues in the effector domain are partly embedded in the membrane [26]. Thirdly, several observations point to major differences in the modes of action of MARCKS and MRP. For example, and in contrast to MARCKS, MRP is found on phagocytotic cups but was not detected on mature phagosomes [25,27]. Finally, the effect of phosphorylation and CaM on the interactions of MRP with membranes has not been hitherto investigated at all. These observations stress the need for characterizing MRP.

In order to gain insight into the aspects mentioned above, we have investigated the interactions of MRP with sucrose-loaded phospholipid vesicles. In particular, the effects of myristoylation, phospholipid composition, CaM binding, and PKC-dependent phosphorylation on the binding of MRP to vesicles were investigated. We show that MRP binds to negatively-charged phospholipid vesicles with a significantly lower affinity than reported for MARCKS. Also CaM and phosphorylation do not dramatically decrease the binding of MRP.

MATERIALS AND METHODS

Materials

The plasmid pET3dF52M1 containing the *mrp* gene was a gift from Perry Blackshear (Duke University Medical Center, Durham, NC, U.S.A.). The plasmid pBB131NMT containing the gene coding for NMT was a gift from Jeffrey Gordon (Washington University, School of Medicine, St. Louis, MO, U.S.A.). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) were from Avanti Polar Lipids (Alabaster, AL, U.S.A.).

Proteins and peptides

The unmyristoylated (unmyr) and myristoylated (myr) forms of mouse MRP were expressed in *Escherichia coli* and purified as described previously [17]. MRP can be stoichiometrically myristoylated by coexpressing NMT in bacteria, judged by radioactive labelling with [^{3}H]myristate and by a shift in the migration of the MRP band on SDS/polyacrylamide gels. During purification, myrMRP is retained on Phenyl-Sepharose in the presence of 3 M NaCl whereas unmyrMRP is not, ensuring that the myristoylated protein is not contaminated with traces of unmyrMRP [17]. The concentration of MRP was determined by amino-acid analysis. The catalytic subunit of PKC (PKM) from rat brain was purchased from Calbiochem (La Jolla, CA, U.S.A.). Bovine brain CaM and dansylated CaM were from Sigma. The proteins were stored in 10 mM Mops/NaOH (pH 7.4)/0.1 mM EGTA (buffer A) at −80 °C.

Note that unmyrMRP, as well as myrMRP, appears as a double band on SDS/polyacrylamide gels (see Figures 1–4). This phenomenon is not due to an artefactual processing of the protein in *E. coli*, since a double band is also observed in Western blots of macrophage extracts [28]. We have so far found no evidence for a co- or post-translational modification which could explain this pattern. Since MRP is acidic and migrates anomalously on SDS/polyacrylamide gels, we hypothesize that the double band represents a conformational equilibrium resulting from incomplete binding of SDS to this protein.

Binding of MRP to sucrose-loaded vesicles

The assay of binding of MRP to sucrose-loaded vesicles was adapted from procedures described previously [17,29]. A suspension containing 15 mg POPC or 15 mg of a mixture of POPC and POPG (4:1, w/w) was dried and resuspended in 1.3 ml of buffer B [10 mM Mops (pH 7.4)/0.1 mM EGTA/1 mM dithiothreitol (DTT)/170 mM sucrose]. The lipid suspensions were freeze–thawed 5 times in liquid N_2 and extruded 10 times through a 100-nm polycarbonate filter (Costar Scientific Corporation, Cambridge, MA, U.S.A.). The vesicles were diluted with 5 vol. of buffer C [10 mM Mops (pH 7.4)/0.1 mM EGTA/1 mM DTT/100 mM NaCl] and centrifuged at 100000 *g* and 22 °C for 60 min (Optima TLX Ultacentrifuge, TLA-100.3 rotor and 1.5 ml polyallomer microfuge tubes from Beckman). The supernatant was removed and the vesicles were resuspended by pipetting with 250 μl of buffer C. The lipid concentration was measured by phosphate analysis [30] and adjusted to 12 mM phosphate with buffer C.

A solution containing 1 μM MRP in buffer D [10 mM Mops (pH 7.4)/0.1 mM EGTA/1 mM DTT/100 mM NaCl/0.01 % (v/v) Triton X-100] was prepared and incubated at room temperature for 15 min. To remove possible MRP aggregates, the solution was precleared by centrifugation at 100000 *g* and 22 °C for 60 min (TL-100.3 rotor and 1.5 ml polyallomer microfuge tubes). The supernatant was used as an MRP stock solution.

For the binding assay, 20 μl of the stock solution containing 50 ng of MRP was mixed with increasing concentrations of lipid vesicles in 300 μl polycarbonate centrifuge tubes (Beckman). The final volume was adjusted to 200 μl with buffer C (giving an MRP concentration of 100 nM) and the solutions were incubated for 60 min at room temperature. No differences were found if this incubation time was doubled. Membrane-bound MRP was separated from the free protein by centrifugation at 100000 *g* and 22 °C for 60 min (TLA-100 rotor, Beckman). After centrifugation, the supernatant was removed and 120 μl of a solution containing buffer C and sample buffer [1 % (w/v) SDS/50 % (v/v) glycerol/250 mM Tris/HCl (pH 6.8)/0.1 % (w/v) Bromphenol Blue/5 % (v/v) mercaptoethanol] (4:1; v/v) was added to the pellet. To solubilize the vesicles, the samples were incubated overnight at 4 °C and subsequently mixed by pipetting. Note that under the conditions used in this assay, MRP neither adsorbed significantly to the wall of the centrifugation tubes nor pelleted during centrifugation in the absence of lipids.

To estimate the extent of binding of MRP to the lipid vesicles, 10 μl of the solutions containing the pellets (maximum amount, 33 ng MRP) were loaded onto an SDS/polyacrylamide gel (12.5 % acrylamide). MRP (33, 25, 17 and 8 ng, corresponding to 100, 75, 50, and 25 % of the total amount of protein in each sample respectively) were also loaded onto the gel as a standard for quantification. After electrophoresis, MRP was transferred to nitrocellulose membranes and detected by Western blotting using a polyclonal rabbit anti-MRP antibody at a dilution of 1/2000. The antibody recognizes myrMRP and unmyrMRP as well as phospho- and dephospho-MRP. A goat anti-rabbit horseradish peroxidase-conjugated IgG (Sigma) was used at a dilution of 1/5000 as the secondary antibody. MRP was detected by exposure of the membranes on a Biomax MR film (Kodak) using enhanced chemiluminescence (ECL) (Amersham). The films were scanned using a personal densitometer (Molecular Dynamics) and the MRP signals were quantified by volume integration with local background substraction at 100-μm resolution. The inset in Figure 1(B) shows that the ECL signal increased linearly with the amount of MRP in a range corresponding to 25–100 % of the total MRP fraction used in each sample. Fitting this data with a linear regression gave a standard function which was used to calculate the fraction of MRP associated with the vesicles as a function of the phospholipid

concentration. The fraction of MRP associated with the vesicles (f_b), was defined as the ratio of density (at a given lipid concentration) to the density of the total amount of protein.

Phosphorylation of MRP

unmyrMRP and myrMRP were phosphorylated for 3 h at 30 °C in a volume of 400 μl. The solutions contained 3–5 mM MRP, 3 nM PKM, 6 mM $MgCl_2$, 100 mM NaCl, 0.1 mM ATP, 0.2 mM $CaCl_2$, 0.1 mM EGTA and 10 mM Mops, pH 7.4. The extent of modification was estimated by phosphorylating 20 μl aliquots in the presence of 0.5 μCi [γ-^{32}P]ATP as described previously [31]. The stoichiometry of phosphorylation was determined by exposing the gels containing phosphorylated MRP together with a nitrocellulose membrane on which known amounts of [γ-^{32}P]ATP (0–200 pmol in 5 μl) were spotted. The conditions (amount of radioactivity and exposure time) were chosen so that the signals from phosphorylated MRP and from the calibration curve were in the linear range. Although phosphopeptide analysis showed that two serine residues are modified in MRP [9,31] we were able to incorporate only 1.39 ± 0.15 mol phosphate/mol of myrMRP (n = 4) and 0.93 ± 0.15 mol phosphate/mol of unmyrMRP (n = 4). For comparison, a value of 2.5 mol phosphate/mol of protein was reported for unmyrMRP [9]. This value should be reduced to 1.6 mol phosphate/mol of protein since amino-acid analysis has shown that the Lowry assay overestimates the concentration of MRP by a factor of 1.6 [31].

For the binding of phosphorylated MRP to lipid vesicles, the solutions were diluted to 1 μM with buffer D and precleared by centrifugation as described above. In both studies, control experiments were performed by omitting PKM from the reaction solution.

RESULTS

Binding of myrMRP to vesicles containing 20% POPG

We had previously investigated the binding of MRP to 100-nm sucrose-loaded vesicles [17]; the amount of MRP associated with the vesicles was estimated by scanning densitometry of Coomassie-Blue-stained SDS/polyacrylamide gels. Since a relatively high concentration of MRP was required to perform a quantitative analysis (1 μM), the effect of phosphorylation as well as of CaM on the interaction of MRP with membranes could not be investigated because of the limited amounts of protein available. In the present report, the concentration of MRP was lowered ten-fold (100 nM) and the amount of protein associated with the vesicles was estimated by Western blotting using a polyclonal MRP antibody.

Figure 1 shows the binding of 100 nM myrMRP to POPC:POPG (4:1) vesicles. In the presence of increasing concentrations of phospholipid, an increasing fraction of myrMRP becomes associated with the vesicles. As the accessible phospholipid concentration approaches 1 mM most of the protein binds to the vesicles. Using a radius of 50 nm for the vesicles and an area of 0.7 nm² for a phospholipid, the number of lipid molecules per vesicle equals 90000, of which approximately half are accessible. Hence, at this concentration (1 mM), the concentration of vesicles is 22 nM, to which 100 nM protein is bound, i.e. approximately five proteins per vesicle. Since one vesicle has a surface area of 31000 nm², this implies that protein–protein interactions will be negligible in the binding. Even at the start of titration where, for example, 20% of the total MRP is bound to the vesicles, corresponding to 20 nM MRP bound to 28 μM lipid, we have only 33 proteins per vesicle. This still means that

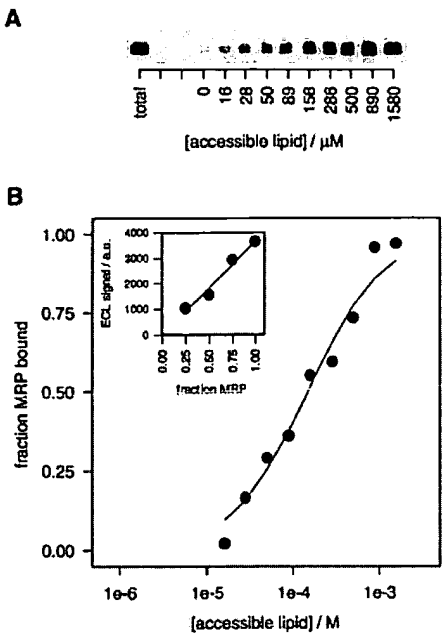

Figure 1 Binding of myrMRP to POPC:POPG (4:1) sucrose-loaded vesicles

Solutions containing 100 nM MRP were incubated with increasing amounts of 100 nm sucrose-loaded POPC:POPG (4:1) vesicles for 1 h at 22 °C. Membrane-bound myrMRP was separated from the free protein by centrifugation at 100000 *g* for 1 h at 22 °C. (**A**) Western-blot analysis of MRP associated with the vesicles as a function of the accessible phospholipid concentration. Note that the accessible lipid concentrations, shown at the bottom of the panel, were chosen to obtain regular spacing on a logarithmic scale (see **B**). The spot on the left labelled 'total' contained 33 ng of MRP, which is equivalent to the total amount of protein used in each sample. (**B**) Fraction of MRP associated with the vesicles as a function of the phospholipid concentration. The standard curve shown in the inset was obtained by linear-regression analysis and was used to calculate the fraction of myrMRP bound to the vesicles [Linear regression: $y = ax + b$, where y is the ECL signal, x is the fraction MRP, a (3696 ± 444) and b (−19 ± 304) are the fitted parameters]. The accessible phospholipid concentrations are shown on a logarithmic scale. The data in (**B**) were then fitted to eqn. (2) to obtain K_p (6.7 ± 0.7 × 10³ · M⁻¹) or K_d^{eff} (149 ± 16 μM). $[L]_{50}$, the accessible phospholipid concentration at which 50% myrMRP is associated with the vesicles, was estimated directly by interpolation of the data (139 μM).

approx. 900 nm² of lipid surface per bound protein on average. Kim et al. [13] have considered MARCKS–membrane association as an equilibrium involving protein, P and lipid, L:

$$P_f + L \rightleftharpoons P_L$$

where P_f and P_L denote free (dissolved) and membrane-bound protein respectively, and define a corresponding molar partition coefficient K_p (M^{-1}) according to:

$$K_p = p_L/[L]p_f \quad (1)$$

where [L] is the accessible lipid concentration and p denotes protein concentrations. Eqn. (1) can be rewritten as:

$$f_b = K_p[L]/(1 + K_p[L]) \quad (2)$$

where:

$$f_b = p_L/p_{tot} \quad (3)$$

is the fraction of protein bound to the vesicles. Eqn. (2) was fitted to the data (Figure 1B) with K_p as a free parameter. The inverse of K_p, K_d^{eff} (M), is an effective dissociation constant corresponding to the concentration of accessible lipid at which 50% of the MRP is bound to vesicles. Therefore, K_d^{eff} can also be estimated without fitting by determining $[L]_{50\%}$, the concentration of

Table 1 Effect of myristoylation and lipid composition on the affinity of MRP and MARCKS for vesicles

The third column shows experimentally-determined effective dissociation constants (K_d^{eff}) [see eqns. (1) and (2)] describing the binding of different MRP and MARCKS constructs to vesicles of different phospholipid composition. The fourth column shows dissociation constants ($K_d^{co\text{-}op}$) calculated according to eqn. (4). n = number of experiments. *Taken from [13]. †Taken from [12].

Protein	Vesicles	K_d^{eff}	$K_d^{co\text{-}op}$
myrMRP	POPC:POPG (4:1)	137 ± 94 μM [a] ($n = 8$)	1.5–3 μM
myrMRP	POPC	800 ± 200 μM ($n = 3$) (K_A)	
unmyrMRP	POPC:POPG (4:1)	800 ± 100 μM ($n = 3$) (K_B)	
unmyrMRP	POPC	> 5 mM ($n = 4$)	
myrMARCKS	POPC:POPG (4:1)	3 μM*	7 nM
myrMARCKS	POPC	385 μM* (K_A)	
effector peptide of MARCKS	POPC:POPG (4:1)	0.25 μM† (K_B)	
effector peptide of MARCKS	POPC	'little binding'†	

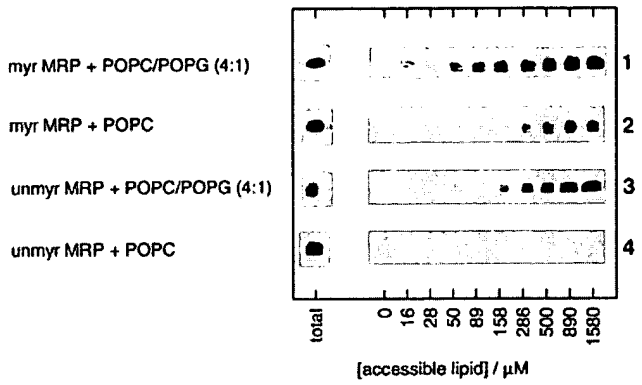

Figure 2 Effect of myristoylation and anionic phospholipids on the binding of MRP to sucrose-loaded vesicles

myrMRP or unmyrMRP (100 nM) was incubated with increasing concentrations of POPC or POPC:POPG (4:1) vesicles. The binding was analysed as described in Figure 1.

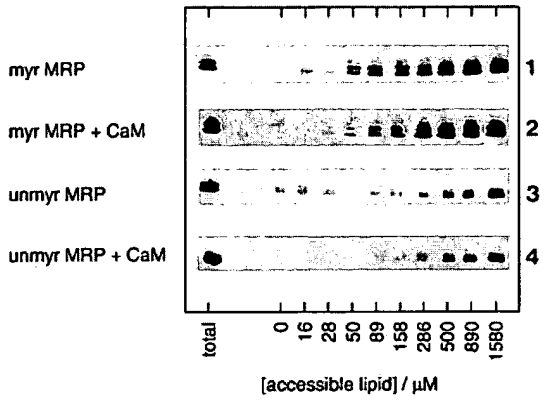

Figure 3 Effect of CaM on the binding of MRP to sucrose-loaded vesicles

Following incubation of myrMRP and unmyrMRP with CaM in the presence of calcium, the solutions were incubated with increasing concentrations of POPC:POPG (4:1) vesicles. The binding was analysed as described in Figure 1. CaM was omitted in controls.

accessible phospholipid required to bind 50% MRP, by interpolation of the data. The values for K_d^{eff}, determined by fitting, are given in Table 1.

The results of the standards and of the binding experiments were generally more scattered compared with the values shown in Figure 1, which explains the relatively large standard errors in K_d^{eff} and $[L]_{50\%}$. Hence care should be taken not to overinterpret the results from experiments in which K_d^{eff} differs by less than a factor of three. As a rule of thumb, we suggest that only differences in binding which are easily detected by visual inspection of the Western blots should be regarded as significant. The data in Figures 2–4, therefore, is shown in the form of Western blots only.

Using this method and bearing in mind the remarks in the previous paragraphs, the roles of myristoylation, lipid composition, CaM and phosphorylation by PKM on the interactions of MRP with vesicles were assessed. The results are illustrated in Figures 2–4.

Effect of myristoylation and lipid composition

Figure 2 compares the binding of myrMRP and unmyrMRP to POPC and POPC:POPG (4:1) vesicles. The second lane in Figure 2 shows that removing anionic phospholipids reduces, but does not abolish, the binding of myrMRP to vesicles. Similarly, removing the myristoyl group significantly reduces, but does not abolish, the binding of MRP to POPC:POPG (4:1) vesicles (lane 3). Lane 4 shows that unmyrMRP does not bind to POPC vesicles. No binding was detected even in the presence of as much as 5 mM accessible lipids (results not shown). K_d^{eff} values are given in Table 1.

Effect of CaM

It has been proposed that one function of MARCKS proteins is to regulate the activity of CaM by complexing the protein in a calcium-dependent manner [4]. This interaction could take place in the cytoplasm or, alternatively, MARCKS proteins might sequestrate CaM at the membrane. In order to understand how MRP, CaM and the membrane interact with each other we have investigated the binding of myrMRP and unmyrMRP to POPC:POPG (4:1) vesicles in the absence and presence of CaM (Figure 3). A three-fold molar excess of CaM was first incubated with myrMRP for 15 min to ensure that most of the MRP was complexed with CaM [31]. The proteins were then incubated with vesicles for 1 h and the binding was analysed as in Figure 1. A visual inspection of lanes 1 and 2 in Figure 3 revealed no significant effect of CaM on the binding of MRP to POPC:POPG (4:1) vesicles. This was independent of the order in which the proteins were added; addition of CaM to solutions in which myrMRP and vesicles were first incubated did not influence the

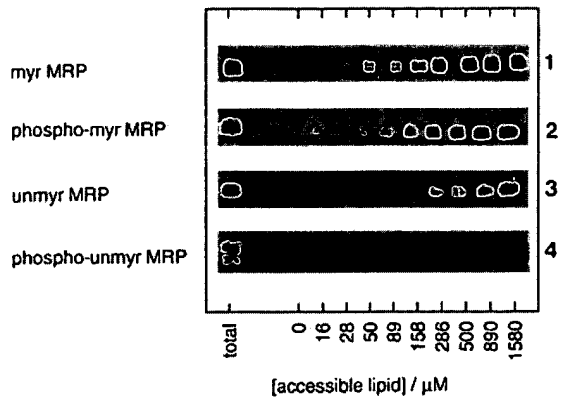

Figure 4 Effect of phosphorylation on the binding of MRP to sucrose-loaded vesicles

myrMRP and unmyrMRP (5 mM) were phosphorylated with PKM. MRP was then diluted to a final concentration of 100 nM and incubated with increasing concentrations of POPC:POPG (4:1) vesicles. The binding was analysed as described in Figure 1. As controls, myrMRP and unmyrMRP were incubated in phosphorylation buffer in the absence of PKM. The proteins were phosphorylated to the following extent; 1.26 mol phosphate/mol of myrMRP and 0.86 mol phosphate/mol of unmyrMRP.

binding (results not shown). When CaM was replaced by dansylated CaM to allow detection of the protein by fluorescence spectroscopy, > 93% of CaM could be detected in the supernatant of each of the samples titrated with vesicles (results not shown).

In an equilibrium situation, the fraction of MRP associated with the vesicles in the presence of CaM depends on the affinity of MRP for vesicles as well as for CaM [13]. The binding of MRP to vesicles cannot, therefore, be analysed by a simple partitioning mechanism. The effective dissociation constant determined by means of eqn. (2) was, therefore, designated K_d^{obs} for the experiments performed in the presence of CaM. From the results of the fitting we estimated that, in the presence of 300 nM CaM, 100 nM myrMRP bound to POPC:POPG (4:1) vesicles with a K_d^{obs} of 144 ± 49 μM ($n = 4$). In the control experiments, in which CaM was omitted, myrMRP bound with an effective dissociation constant of 37 ± 18 μM ($n = 4$), suggesting that CaM might slightly inhibit the binding of myrMRP. However, replacing 0.1 mM EDTA (Figure 2, lane 1) with 0.1 mM $CaCl_2$ (Figure 3, lane 1) decreased the average K_d^{eff} from 137 μM to 37 μM, suggesting that calcium alone might increase the affinity of myrMRP for POPC:POPG (4:1) vesicles. Since MRP is overall an acidic protein (pI = 4.4), this phenomenon could be an example of calcium stabilizing MRP at the surface of a membrane of like charge by diminishing the electrostatic repulsion between the protein and the membrane surface [32]. These differences in K_d^{eff} should, however, be taken merely as indicative, since they are small compared with the accuracy of the method. Finally, the results shown in Figure 3 (lanes 3 and 4) demonstrate that 300 nM CaM had practically no observable effect on the binding of 100 nM unmyrMRP to POPC:POPG vesicles.

Effect of phosphorylation

Visual inspection of the amount of MRP associated with the vesicles in the first two lanes of Figure 4 shows that phosphorylation had no dramatic effect on the binding of myrMRP to POPC:POPG (4:1) vesicles. In agreement with this finding, increased amounts of protein were not detected in the supernatants of the samples containing the phosphorylated protein compared with the non-phosphorylated protein (results not shown).

Because of the limited amounts of PKM available, only a few experiments could be performed (two for myrMRP and three for unmyrMRP). In the first experiment, results shown in Figure 4, 1.26 nmol phosphate/mol of myrMRP were incorporated and a K_d^{eff} of 454 μM was estimated for the binding of the phosphoprotein to POPC:POPG (4:1) vesicles. In a second experiment, 1.36 nmol phosphate/mol of myrMRP was incorporated and the K_d^{eff} was estimated to be 270 μM. Although the data suggest that phosphorylation slightly decreases the affinity of myrMRP for POPC:POPG (4:1) membrane (compared with the average value of 137 μM for the non-phosphorylated protein), this difference is not sufficiently large to be considered significant.

Comparison of lanes 3 and 4 in Figure 4 reveals, however, that the binding of unmyrMRP to POPC:POPG (4:1) vesicles is clearly reduced by phosphorylation and no detectable protein is associated with the pellet, even in the presence of 1.58 mM accessible lipid (hence $K_d^{eff} > 1.58$ mM; $n = 3$).

DISCUSSION

Binding of myrMRP to phospholipid vesicles

The effective dissociation constant previously determined for the binding of myrMRP to vesicles containing 80% egg lecithin and 20% brain phosphatidylserine (K_d^{eff} = 120–200 μM) [17] agrees well with the value reported in the present work for POPC:POPG (4:1) vesicles ($K_d^{eff} = 137 \pm 94$ μM). In contrast to egg lecithin and brain phosphatidylserine, which have a heterogeneous chemical composition, synthetic phospholipids such as POPC and POPG have a well defined length and degree of saturation of the acyl chains (16:0–18:1). We conclude, therefore, that these parameters do not significantly modulate the interactions of MRP with vesicles. Furthermore, since MRP has similar affinities for vesicles containing either POPS or POPG (results not shown), electrostatic effects, rather than chemical ones, mediate the binding of the effector domain of MRP to negatively-charged phospholipid vesicles; this behaviour was also reported for MARCKS [13]. Note that we found a significantly lower affinity of myrMRP for egg lecithin vesicles ($K_d^{eff} > 10$ mM) [17] compared with POPC vesicles ($K_d^{eff} = 800$ μM).

Co-operativity between the myristoyl group and the effector domain

Figures 1 and 2 show that both insertion of the myristoyl group in the bilayer and electrostatic interactions of the effector domain with anionic membrane surfaces influence the binding of 100 nM MRP to vesicular membranes. Furthermore, the significantly higher affinity of myrMRP for POPC:POPG (4:1) vesicles compared with the affinity of myrMRP for POPC vesicles and unmyrMRP for POPC:POPG (4:1) vesicles prompts an examination of whether the two domains act co-operatively; co-operative behaviour has been inferred previously for Src [33] and MARCKS [13]. McLaughlin and Aderem [18] have proposed the so-called 'two balls and string' model to account for this phenomenon. Tethering of one domain to the membrane confines the second domain to a hemisphere on the surface of the membrane, thus increasing the probability that the second domain will find the membrane. The reduction in dimensionality results in a dissociation constant for the intact molecule, $K_d^{co\text{-}op}$ (M), which is expressed as follows [18]:

$$K_d^{co\text{-}op} \approx K_A K_B (r/\alpha) \quad (4)$$

where K_A (M) and K_B (M) are the effective dissociation constants of the individual domains [K_A, binding of the myristoyl moiety to POPC vesicles; K_B, binding of unmyristoylated MRP to POPC:POPG (4:1) vesicles]; r (nm) is the distance between the domains; α (4 nm · M) is a correction factor which transforms the units of one of the dissociation constants from molar (M) to a reciprocal distance (nm^{-1}) [15]. In the absence of a known structure for MRP, r is difficult to estimate: the 'string' connecting the modules must comprise about 100 residues. Since MRP is a rod-shaped protein with a long axis ranging between 13 and 19 nm [31], and since the effector domain is in the middle of the amino acid sequence of MRP, we can estimate r as approx. 10 nm. Hence the factor $r/\alpha = 2.5\ M^{-1}$. Using the K_A and K_B values from Table 1, we calculate that $K_d^{co\text{-}op} \approx 1.5\ \mu M$, two orders of magnitude lower than the experimentally-determined value of 137 μM. Note that the parallel calculation for MARCKS cannot be carried out exactly, since the required value for K_B has not been measured for the intact unmyristoylated protein but for the effector peptide ($K_B = 0.25\ \mu M$) [12]. Using this value, and bearing in mind that MARCKS is a larger protein ($r = 25$ nm) [13] we calculated that $K_d^{co\text{-}op} = 7$ nM, and is almost three orders of magnitude smaller than the measured value (Table 1). The inadequacy of the simple co-operative model suggests that electrostatic repulsion between negatively-charged amino acid residues adjacent to the effector domain and the vesicle phospholipids could well weaken the interaction of the effector domain with the vesicles. This has been proposed for MARCKS, but not demonstrated experimentally [13].

Comparison of MRP and MARCKS

The affinity of myrMRP for POPC:POPG (4:1) vesicles ($K_d^{eff} = 137\ \mu M$) is more than one order of magnitude weaker than that reported for the binding of myristoylated MARCKS to vesicles of the same size and lipid composition ($K_d^{eff} = 3\ \mu M$) [13]. Since myrMRP ($K_d^{eff} = 0.8$ mM) (Figure 2) and myristoylated MARCKS ($K_d^{eff} = 0.4$ mM) [13] have similar effective dissociation constants for POPC vesicles, the significantly higher affinity of myristoylated MARCKS for POPC:POPG (4:1) vesicles suggests that the electrostatic interaction of MRP with negatively-charged membranes is weaker than for MARCKS. Two explanations might be advanced to account for this difference: (1) the extent of the interaction can be expected to depend on the distribution of negatively-charged residues on the protein surface; these distributions are not known but there is no reason to believe that they are the same for MARCKS and MRP; (2) although its amino acid sequence is otherwise highly conserved, the middle of the effector domain of MARCKS contains a serine residue which is replaced by a proline in MRP [34]. This mutation is likely to effect the structure of the effector domain and, consequently, its interaction with acidic phospholipids. That the affinity of unmyrMRP for POPC:POPG (4:1) vesicles is three orders of magnitude lower than that reported for the peptide corresponding to the effector domain of MARCKS ($K_d^{eff} = 0.2\ \mu M$) [12] supports either of these hypotheses.

MARCKS has recently been shown to associate with microdomains rich in anionic phospholipids [35]. The affinity of the effector domain for vesicles depends on the fraction of anionic phospholipid present [12] and the two proteins may well respond differently to this parameter. Hence MARCKS and MRP would have different affinities for such microdomains *in vivo*. In this respect, it is intriguing to note that MRP and MARCKS appear to have a different subcellular localization pattern *in vivo* (see the Introduction). A direct comparison of the relative affinities of the unmyristoylated forms of MRP and MARCKS and of their effector domains for different POPC:POPG ratios would help to clarify these hypotheses.

Effect of CaM

Although the CaM/MRP ratio was chosen to make sure that most of the MRP would be complexed to CaM in the absence of POPC:POPG (4:1) vesicles [31] our CaM concentration (300 nM) was insufficient to significantly displace MRP already bound to the vesicles or to inhibit the binding of MRP to these vesicles. Taken together, our results demonstrate that, under our experimental conditions, a CaM–MRP complex cannot exist at the surface of POPC:POPG (4:1) vesicles.

Effect of phosphorylation

Phosphorylation decreases the affinity of a peptide corresponding to the effector domain of MARCKS for lipid vesicles containing 20% acidic lipid by a factor of 10^4 [12]. Were phosphorylation to completely inhibit the binding of the effector domain of MRP to POPC:POPG (4:1) vesicles, the affinity of phosphorylated myrMRP for these vesicles should be the same as the affinity of unphosphorylated myrMRP for neutral POPC vesicles. Consequently, the effect of phosphorylation on the affinity of myrMRP for POPC/POPG (4:1) vesicles will be difficult to detect, since phosphorylation should not decrease the affinity of myrMRP for POPC:POPG (4:1) vesicles by more than a factor of about six. In fact, although scanning densitometry of the data reveals that phosphorylation might indeed slightly decrease the affinity of myrMRP for POPC:POPG (4:1) vesicles, we did not observe significant inhibition: phospho-myrMRP still bound more strongly to POPC:POPG (4:1) vesicles than myrMRP did to POPC vesicles. One might argue that the lack (or the low level) of inhibition might be due to incomplete phosphorylation (see the Materials and methods section). This is however unlikely since, although slightly less phosphate was incorporated into unmyrMRP compared with myrMRP, we found that phosphorylation dramatically inhibited the binding of unmyrMRP to POPC:POPG (4:1) vesicles. The arguments proposed for myrMRP can also be used to predict the effect of phosphorylation on unmyrMRP; phospho-unmyrMRP should bind to POPC:POPG (4:1) vesicles to the same extent as unmyrMRP does to POPC. Since unmyrMRP does not bind to POPC vesicles it was not surprising to find that phosphorylation had a significant effect on the binding of unmyrMRP. The physiological relevance of this last observation should be questioned since the presence of unmyrMRP in cells has not been reported so far. Note, however, that a pool of non-myristoylated MARCKS is present in brain cytosolic extracts [36] and that demyristoylation activity has been detected in cytosolic fractions of brain synaptosomes [37].

Numerous studies *in vitro* and *in vivo* propose that phosphorylation regulates the subcellular localization of MARCKS by decreasing its affinity for membranes (see the Introduction). Although phosphorylation does reduce the affinity of unmyrMRP for POPC:POPG vesicles, the results presented here indicate that phosphorylation has only a mild effect on the affinity of myrMRP for membranes. Our observations, therefore, raise the possibility that, in contrast to MARCKS, phosphorylation does not directly regulate the subcellular localization of MRP. Note that this hypothesis does not exclude the possibility that phosphorylation regulates the interaction of MRP with other membrane-bound proteins, and by this means its subcellular localization.

This research was partly supported by the Swiss National Science Foundation (grant 3100-042045.94 to G. Schwarz). We are grateful to Christoph Stürzinger for purifying MRP.

REFERENCES

1 Stumpo, D. J., Bock, C. B., Tuttle, J. S. and Blackshear, P. J. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 944–948
2 Wu, M., Chen, D. F., Sasaoka, T. O. and Tonegawa, S. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 2110–2115
3 Aderem, A. A., Albert, K. A., Keum, M. M., Wang, J. K. T., Greengard, P. and Cohn, Z. A. (1988) Nature (London) **332**, 362–364
4 Blackshear, P. J. (1993) J. Biol. Chem. **268**, 1501–1504
5 Li, J. and Aderem, A. A. (1992) Cell **70**, 791–801
6 Lobach, D. F., Rochelle, J. M., Watson, M. L., Seldin, M. F. and Blackshear, P. J. (1993) Genomics **17**, 194–204
7 Rosen, A., Nairn, A. C., Greengard, P., Cohn, Z. A. and Aderem, A. A. (1989) J. Biol. Chem. **264**, 9118–9121
8 Graff, J. M., Young, T. N., Johnson, J. D. and Blackshear, P. J. (1989) J. Biol. Chem. **264**, 21818–21823
9 Verghese, G. M., Johnson, J. D., Vasulka, C., Haupt, D. M., Stumpo, D. J. and Blackshear, P. J. (1994) J. Biol. Chem. **269**, 9361–9367
10 Hartwig, J. H., Thelen, M., Rosen, A., Janmey, P. A., Nairn, A. C. and Aderem, A. A. (1992) Nature (London) **356**, 618–622
11 Taniguchi, H. and Manenti, S. (1993) J. Biol. Chem. **268**, 9960–9963
12 Kim, J., Blackshear, P. J., Johnson, J. D. and McLaughlin, S. (1994) Biophys. J. **67**, 227–237
13 Kim, J., Shishido, T., Jiang, X., Aderem, A. A. and McLaughlin, S. (1994) J. Biol. Chem. **269**, 28214–28219
14 Graff, J. M., Gordon, J. I. and Blackshear, P. J. (1989) Science **246**, 503–506
15 Peitzsch, R. M. and McLaughlin, S. (1993) Biochemistry **32**, 10436–10443
16 Swierczynski, S. L. and Blackshear, P. J. (1995) J. Biol. Chem. **270**, 13436–13445
17 Vergères, G., Manenti, S., Weber, T. and Stürzinger, C. (1995) J. Biol. Chem. **270**, 19879–19887
18 McLaughlin, S. and Aderem, A. A. (1995) Trends Biochem. Sci. **20**, 272–276
19 Thelen, M., Rosen, A., Nairn, A. C. and Aderem, A. A. (1991) Nature (London) **351**, 320–322
20 Wang, J. K. T., Walaas, S. I., Sihra, T. S., Aderem, A. A. and Greengard, P. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 253–2256
21 Rosen, A., Keenan, K. F., Thelen, M., Nairn, A. C. and Aderem, A. A. (1990) J. Exp. Med. **172**, 1211–1215
22 Vergères, G., Manenti, S. and Weber, T. (1995) in Signaling Mechanisms: from Transcription Factors to Oxidative Stress, vol. H 92, NATO ASI Series (Packer, L. and Wirtz, K. W. A., eds.), pp. 125–138, Springer Verlag, Berlin
23 James, G. and Olson, E. N. (1989) J. Biol. Chem. **264**, 20928–20933
24 Byers, D. M., Palmer, F. B. S. C., Spence, M. W. and Cook, H. W. (1993) J. Neurochem. **60**, 1414–1421
25 Allen, L. A. H. and Aderem, A. A. (1995) J. Exp. Med. **182**, 829–840
26 Qin, Z. and Carafiso, D. S. (1996) Biochemistry **35**, 2917–2925
27 Zhu, Z., Bao, Z. and Li, J. (1995) J. Biol. Chem. **270**, 7652–17655
28 Aderem, A. A., Keum, M. M., Pure, E. and Cohn, Z. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5817–5821
29 Hope, M. J., Bally, M. B., Webb, G. and Cullis, P. R. (1985) Biochim. Biophys. Acta **812**, 55–65
30 Böttcher, C. J. F., van Gent, C. M. and Fries, C. (1961) Anal. Chim. Acta **24**, 203–204
31 Schleiff, E., Schmitz, A., McIlhinney, R. A. J., Manenti, S. and Vergères, G. (1996) J. Biol. Chem. **271**, 26794–26802
32 van Dulm, P., Norde, W. and Lyklemsa, J. (1981) J. Colloid Interface Sci. **82**, 77–82
33 Buser, C. A., Sigal, C. T., Resh, M. D. and McLaughlin, S. (1994) Biochemistry **33**, 13093–13101
34 Aderem, A. A. (1992) Cell **71**, 713–716
35 Glaser, M., Wanaski, S., Buser, C. A., Boguslavsky, V., Rashidzada, W., Morris, A., Rebecchi, M., Scarlata, S. F., Runnels, L. W., Prestwich, G. D., et al. (1996) J. Biol. Chem. **271**, 26187–26193
36 McIlhinney, R. A. J. and McGlone, K. (1990) Biochem. J. **271**, 681–685
37 Manenti, S., Sorokine, O., Van Dorsselaer, A. and Taniguchi, H. (1994) J. Biol. Chem. **269**, 8309–8313

Received 1 September 1997/9 October 1997; accepted 27 October 1997